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TITLE: Targeted Therapies for Myeloma and Metastatic Bone Cancers

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CONTRACTING ORGANIZATION: Southwest Research Institute
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Neal Vail, Ph.D.

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14. ABSTRACT

Multiple myeloma is the second most common adult hematologic malignancy accounting for 1-2% of cancer-related deaths with 80% of these patients suffering devastating and progressive bone destruction. New treatment strategies are of urgent and vital importance. Several proteasome inhibitors are effective against both human and murine myeloma cells in culture and some have been shown to affect osteoblast differentiation and bone formation in rodents. However, as with any proteasome inhibitor, there are serious concerns over their potential systemic effects and toxicity. There is need to preferentially deliver these and other drugs to the bone microenvironment. The scope of this project is to determine, in preclinical studies, the potential of skeletally targeted PIs as an efficacious and selective treatment for myeloma. The program hypothesis is that bone-targeting nanocarriers can preferentially accumulate in the skeleton and locally release PIs to impair the capacity of myeloma cells to survive and grow in vivo, thereby reducing the formation and growth of tumor-induced lytic bone lesions. Proteasome inhibitors are not selective to bone and their therapeutic-toxic window may be narrow when administered systemically. Targeted bone delivery has potential to reduce systemic exposure, increase efficacy in the bone environment, and the opportunity to reverse catastrophic disease processes.

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Introduction

The goal of this project is to determine, in preclinical studies, the potential of skeletally targeted proteasome inhibitors as an efficacious and selective treatment for myeloma. We have found that several proteasome inhibitors are effective against both human and murine myeloma cells in culture. However, as with any proteasome inhibitor, there are serious concerns over the potential systemic effects and toxicity. Our hypothesis is that bone-targeting nanocarriers can preferentially accumulate in the skeleton and locally release proteasome inhibitors to impair the capacity of myeloma cells to survive and grow in vivo, thereby reducing the formation and growth of tumor-induced lytic bone lesions. Proteasome inhibitors are otherwise not selective to bone and their therapeutic-toxic window may be narrow when administered systemically. The scope of this project is to validate our hypothesis. The major tasks are: 1. Formulate and characterize drug-containing, bone-targeting nanocapsules; 2. Determine the in vivo biodistribution of bone-targeting nanocapsules; and, 3. Evaluate the efficacy of bone-targeted delivery of proteasome inhibitors on myeloma tumor progression using the 5TGM1 murine model of myeloma. The outcomes of this research will be significant. The study will demonstrate the preferential biodistribution of nanocarriers specifically designed to target and adhere to bone matrices. It will further show that these same nanocapsules can selectively deliver a specific and potent proteasome inhibitor to skeletal sites to act as an anti-myeloma agent. Targeted bone delivery has several potential benefits, including reduced systemic exposure, increased efficacy in the targeted microenvironment, and the ultimate opportunity to reverse catastrophic disease processes. Furthermore, targeted delivery to bone has several additional significant application opportunities in the areas of osteoporosis, fracture healing, cartilage repair, and tissue engineering.

Body

The project is broken down into the following tasks:

1. Formulate and characterize drug-containing, bone-targeting nanocarriers
2. Determine the in vivo biodistribution of bone-targeting nanocarriers
3. Demonstrate the efficacy of bone-targeted delivery of proteasome inhibitors on myeloma tumor progress

Task 1 was scheduled to occur during the first year of the project, Task 2 during the second year with some carry over from Task 1, and Task 3 is scheduled to occur during the last year and one-half of the project. Therefore, in this first annual report, there are no technical accomplishments to be reported for Tasks 2 and 3.

Task 1 is focused on the development of the bone-targeting nanoparticles and is broken down into the following subtasks:

1. Selection of proteasome inhibitors for in vivo studies
2. Formulation and characterization of bone-targeting nanoparticles

3. Demonstration of adhesion of bone-targeting nanoparticles to bone-like substrates in vitro
4. Formulation of proteasome inhibitors into bone-targeting nanoparticles.

After conferring with our collaborator, Dr. I. Ross Garrett at OsteoScreen, Inc., we selected four proteasome inhibitors for study, including PS-341, PS-1, MG-132, and MG-262. PS-341, marketed as Velcade™ (bortezomib) by Millenium Pharmaceuticals, is an FDA-approved treatment for multiple myeloma and a necessary benchmark for assessing the efficacy of bone-targeted therapies. The remaining compounds have been studied experimentally in several studies and have been shown to be potent against myeloma in murine models. Furthermore, some of these compounds have also been shown to be potential bone anabolic agents, which may be beneficial in mitigating or countering the pronounced bone loss concomitant with myeloma lesions. MG-132 was procured from commercial sources for the purposes of nanoparticle formulation development.

The bulk of the work during this year of the project was focused on the development of the bone-targeting nanoparticles. This consisted of the following tasks:

1. Synthesis and/or procurement of bone-targeting ligands.
2. Development of a nanoparticle formulation and preparation protocol
3. Attachment of bone-targeting ligands to nanoparticles

In previous work, we identified two bone-targeting ligands: methylene bisphosphonate (MBP) and aspartic acid oligomers (Asp_n). Asp_n is available commercially in several oligomer lengths, typically with n=4 or 6. It has an amino terminus, convenient for covalent coupling to nanoparticle moieties. MBP does not contain suitable moieties for coupling to nanoparticles. Therefore, we synthesized the analogue amino-MBP using methods described previously by Uludag, et al.[1] and Kantoci, et al.[2] We discovered some discrepancies in these reported syntheses occurring in the initial step of a three step reaction sequence. This step involves the formation of the product with the amine and the two phosphates appropriately protected. The byproduct of this adduct is ethanol, which was reportedly removed by simple distillation. However, we found that the reported distillation conditions removed one of the reactants, which was lower boiling than ethanol, resulting in extremely low yields of the product. Addition of excess reactant to reaction pot only favored the production of an undesired by-product. We subsequently modified the initial synthesis step to include a pressurized distillation to enhance removal of the ethanol. We also found that the initial reaction step produced three different adducts, which required separation by chromatography. We produced sufficient quantities of the amino-MBP for our studies, but it may be beneficial to examine the synthesis more closely to determine if it could be modified to more favor the desired product should sizeable quantities of amino-MBP be needed in the future.

Nanoparticle formulation focused reproducibly preparing appropriately-sized particles from the biodegradable polymer polylactide-co-glycolide (PLGA). We developed two protocols for preparing narrowly-distributed nanoparticles in the size range of about 100-200nm: an emulsion/solvent evaporation technique and nanoprecipitation. Both methods

are robust and yield reproducible results. The emulsion/solvent evaporation method tends to yield larger particles than the nanoprecipitation method. The difference in the two methods lies in the miscibility of the polymer solvent with the continuous aqueous phase. In precipitation the solvent is completely miscible with water. The emulsification method is suitable for encapsulating either hydrophilic or lipophilic drugs, while the precipitation method is suitable only for lipophilic drugs. In both methods, the particle size can be effectively tuned by altering the polymer concentration in the initial organic phase (see Figures 1 & 2).

Once the nanoparticles are formed it is necessary to remove residual solvent and other bi-produces, and concentrate them into small volumes. These stepes turned out to be a very technique sensitive process. Literature commonly reports solvent removal by rotary evaporation. This can be effective and fast provided one uses a low volatility solvent. In the case of insoluble drugs, such as some of the proteasome inhibitors and many experimental drugs, it is necessary to use more aggressive solvents, which are not amenable to rotary evaporation. Particle concentration is commonly done by ultracentrifugation. Here, again, success is very dependent on proper choice of conditions. We spent considerable time optimizing centrifuge conditions to minimize excessive particle agglomeration.

In lieu of solvent evaporation and ultracentrifugation to cleanup the nanoparticles, we examined the method of cross-flow filtration. This is effectively a high-volume dialysis process that we found to be efficient for removing both low- and high-boiling solvents, producing a consistently re-dispersible nanoparticle cake. We are integrating this method into our nanoparticle preparation protocols.

We are interested in using polyethylene glycol-modified (PEG) nanoparticles for their improved circulatory properties. Therefore, we prepared PLGA-block-PEG polymers via a dicyclohexylcarbodiimide (DCC) / N-Hydroxysuccinimide (NHS) coupling reaction. The product structure was confirmed by ^{1}H NMR and the spectrum was consistent with spectra reported by Li, et al., for a similar product.[3]

We incorporated PLGA-b-PEG into our PLGA nanoparticle formulation and studied its effects particle properties. Figure 3 shows the particle size decreases with increasing PEG-b-PLGA content. This is as expected, since the block copolymer serves essentially as a surfactant to reduce and stabilize the initial solvated particle emulsion. Figure 4 shows the zeta-potential is also reduced with increasing PEG-b-PLGA content. PLGA nanoparticles are known to exhibit a negative zeta-potential is aqueous suspension. The addition of PEG to the particle surface appears to shield the intrinsic surface charge thereby reducing the observed zeta-potential. However, the reduced zeta-potential does not appear to detrimentally affect particle stability as would be expected in other particle systems as the zeta-potential approaches zero.

We next examined the attachment of the bone-targeting ligands to the nanoparticles. We proposed doing this using previously reported chemisty by preparing a PLA-b-PEG-ligand block copolymer from a bifunctional PEG.[4] Here we ran into an immediate

technical challenge in that the bifunctional PEG was no longer available from the reported source. This forced us to examine alternative methods to achieving the ligand coupling using different starting materials. These all proved unsuccessful. We geared up to prepare the bifunctional PEG in our laboratory, which was to be significant undertaking. Fortunately, we identified another commercial source who only recently introduced the required bifunctional PEG and we quickly procured a large stock pile. This allowed us to proceed with our originally proposed chemistry for preparing the PLA-b-PEG-ligand copolymer. We found it necessary in preparing this compound to first introduce the ligand attachment moiety to the appropriate end of the bifunctional PEG followed by attachment of the PLA polymer block to the other end of the bifunctional PEG by ring-opening polymerization. We have since produced sufficient quantities of these materials with which to conduct ligand attachment studies.

Due to technical difficulties noted above, we are delayed in the ligand attachment studies, although they are currently in progress. In preparation for these studies and to confirm the affinity of the ligands for bone-like substrates, we conducted in vitro studies to examine their adsorption profiles. With the aid of a Summer high school student supported by a grant through the Project SEED program of the American Chemical Society, we studied the adsorption of a FITC-labeled Asp_4 onto high surface area hydroxyapatite powder. This allowed us to understand the ligand conjugation chemistry and confirmed the adsorption of this ligand onto bone-like substrates (see Figure 5). It further provided method development for pending in vitro adsorption studies using ligand-labeled nanoparticles.

As part of the nanoparticle formulation we needed to consider the stabilization of the particles in frozen or powder form for possible long-term storage so specific formulations for the in vivo studies could stock piled for efficiency. This turned out to be another technical challenge, one that is sparsely addressed in the literature and only recently has begun to garner research interest. Polymer nanoparticle stability is compromised by freezing and lyophilization without the aid of protectants. Instability is manifested by severe aggregation of the nanoparticles and an inability to re-establish the intrinsic particle size and distribution on reconstitution. The solution to this problem is taken from previous work with liposomes, which is further based on the stability of bacterial spores. The stability of PLGA nanoparticles is solved by the addition of mono- and di-saccharides to the particle dispersion prior to cryoprocessing. However, we found numerous discrepancies in the limited literature concerning the actual amounts of sugars required relative to the amount nanoparticles. Nevertheless, we found only moderate amounts sugars, particularly sucrose, were required to maintain stability of PLGA nanoparticles (Figure 6). Particle stability is only further compromised when PEG is added to the formulation, because PEG essentially produces an interpenetrating crystal network that is virtually impossible to breakup once formed (Figure 7). There is no literature on this aspect of particle stabilization to cryoprocessing. After considerable experimentation we found sucrose to be an effective protectant for PEG-containing nanoparticles, although it is important to keep the PEG content of the nanoparticles to a minimum, typically less than about 25% wt. based on total polymer. Since improved circulatory properties can be achieved with only moderate PEG contents, we estimated

based on PEG hydrodynamic radius and typical particle size that PEG contents on the order of 5-10% wt are sufficient to produce an adequate PEG corona about the nanoparticles. We are still confirming this estimate by NMR and zeta-potential studies.

We conducted preliminary work to encapsulate MG-132 into PLGA nanoparticles, although given some of the technical challenges in the nanoparticle formulation noted above and the fact no real work with these compounds is schedule to occur until the in vivo efficacy studies, we decided to postpone further work with these compounds until the early in the second year, coincident with the biodistribution studies. We did to method development on an HLPC analytical method to assay proteasome inhibitor content using a modified protocol for detection of amino acids. This was very effective (data not shown).

Key Research Accomplishments

- Synthesized amino-functionalized methylene bisphosphonate to serve as one of two bone-targeting ligands to be attached to polymer nanoparticles.
- Developed two procedures for preparing nanoparticles and demonstrated their ability to repeatedly produce nanoparticles with narrow distribution in the target particle size range of 100-200nm and smaller, if necessary.
- Synthesized PLGA-b-PEG block copolymers to prepare PEGylated nanoparticles with improved circulatory half-lives.
- Formulated PEGylated nanoparticles using available procedures.
- Synthesized PLA-b-PEG-Maleimide block copolymers to facilitate the attachment of bone-targeting ligands to polymer nanoparticles via thiol coupling.
- Developed methods of stabilizing polymer nanoparticles, with and without PEG modification, against cryoprocessing.
- Confirmed adsorption of selected bone-targeting ligands to bone-like substrates in vitro and established ligand coupling chemistry.
- Selected proteasome inhibitors for upcoming studies.
- Encapsulated model proteasome inhibitor, MG-132.
- Established proteasome inhibitor assay for determining nanoparticle payloads.
- Developed animal protocols for upcoming in vivo studies in Tasks 2 and 3.

Reportable Outcomes

- Abstract submitted to DoD-USAMRMC/PRMRP Military Health Research Forum.
- Invited to present results from this program at talk at the Particles 2006 – Medical/Biochemical Diagnostic, Pharmaceutical, and Drug Delivery Applications of Particle Technology Forum scheduled for May 13 –16, in Orlando, FL.
- Invited to give a guest lecture on nanoparticle drug delivery technology to the Graduate Bioengineering Program at the University of Texas at San Antonio, March, 2006.

- Provided an opportunity for a high school student through the Project SEED Program of the American Chemical Society. The program is geared to expose under-represented and disadvantaged high school students to chemistry to encourage their continued education.
- Hired a PhD Bioengineering student who's thesis will be based on work conducted on this project.
- Subcontract in place with UTHSC for the biodistribution study described in Task 2 scheduled for the coming year.

Conclusions

The completed work positions the project to move into the next task of the project. We can consistently prepare polymer nanoparticles of required size and composition necessary to support other tasks of the project. Technical difficulties encountered during the development of the bone-targeting nanoparticles has delayed the project by about two months, such that Subtask 3 of Task 1 remains to be completed prior to moving forward with the biodistribution studies of Task 2. We have considered conducting an intermediate study to refine the particle compositions to enhance the upcoming biodistribution studies and possibly enhance their outcomes. This would consist of an ex vivo perfusion model to screen nanoparticle compositions to optimize in favor of bone uptake. We have developed a protocol for this study and plan to discuss it with the Contracting Officer.

References

1. Uludag, H, Kousinioris, J, Gao, T, and Kantoci D, "Bisphosphonate conjugation to proteins as a means to impart bone affinity," *Biotechnol. Prog.*, **16**, 258-267 (2000).
2. Kantoci *et. al.*, Synthetic Communications, 26(10), 2037-2043 (1996).
3. Li, YP, et al., *J. Controlled Release*, **71**, 203-211 (2001).
4. T. Verrecchia, G. Spenlehauer, D.V. Bazile, A. Murry-Brelier, Y. Archimbaud, and M. Veillard, "Non-stealth (poly(lactic acid/albumin)) and stealth (poly(lactic acid-polyethylene glycol)) nanoparticles as injectable drug carriers," *J. Controlled Release*, **36**, 49-61 (1995).

Appendix 1
Figures

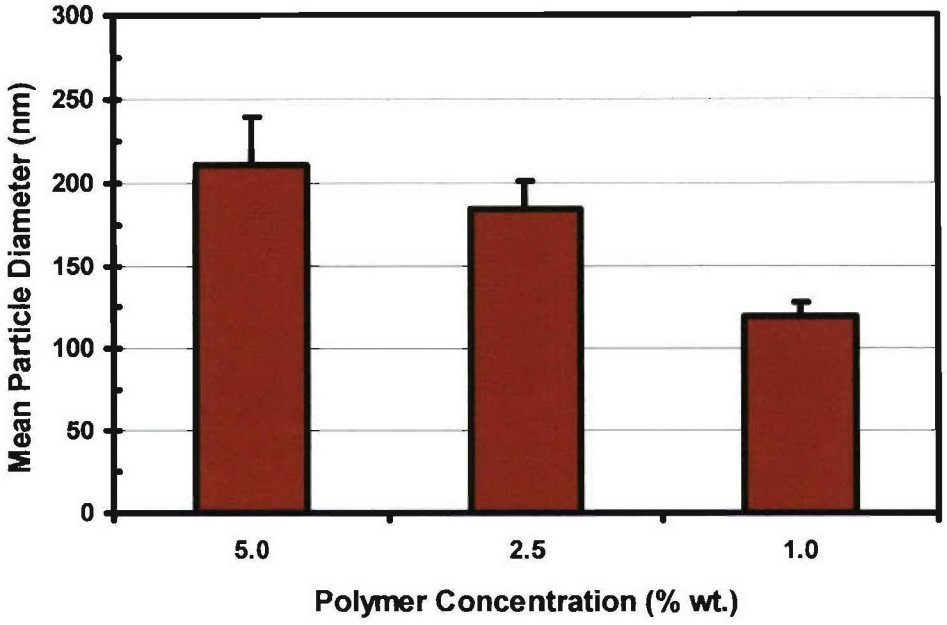


Figure 1. Effect of polymer concentration on the resulting particle size and distribution using emulsion/solvent evaporation.

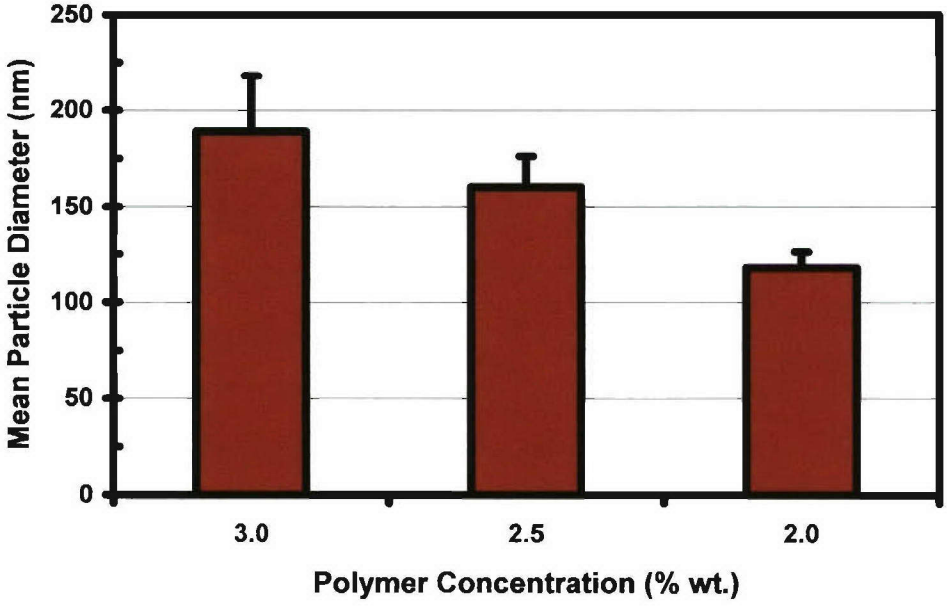


Figure 2. Effect of polymer concentration on the resulting particle size and distribution using nanoprecipitation.

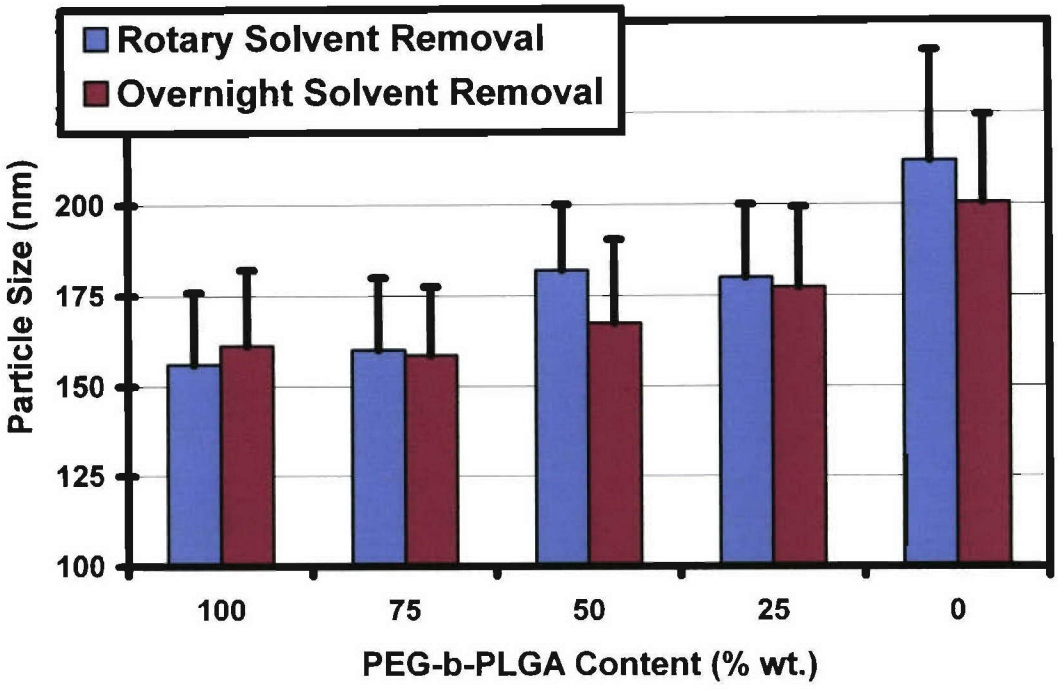


Figure 3. Effect of PEG-b-PLGA content on the particle size and distribution of resulting nanoparticles.

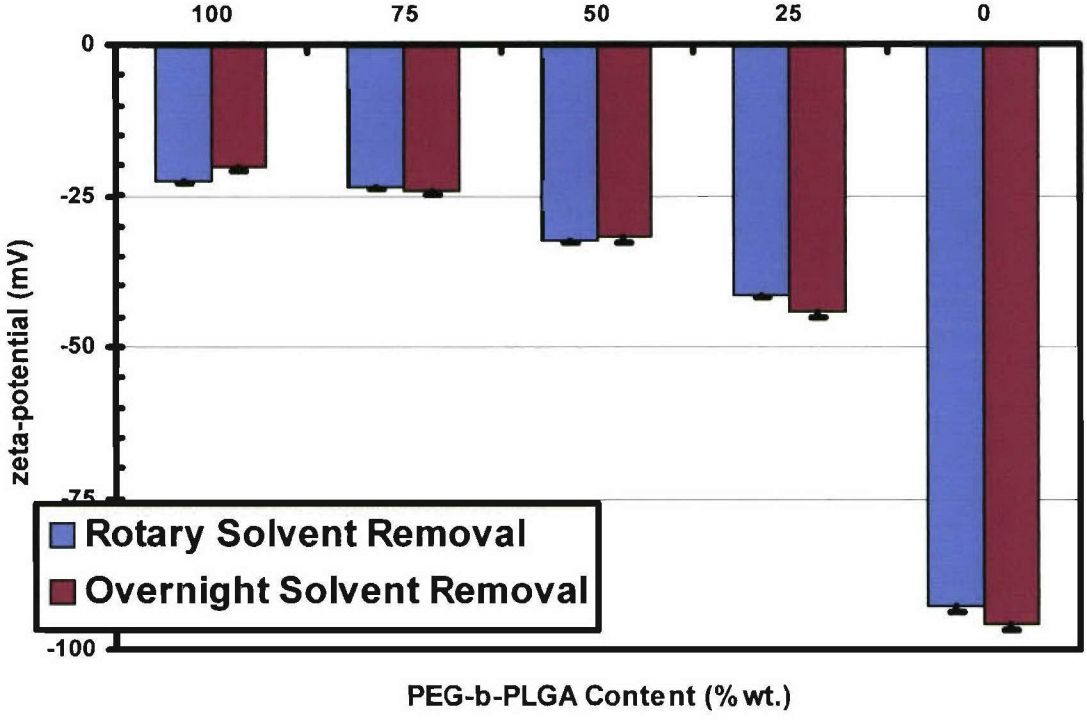


Figure 4. Effect of PEG-b-PLGA content on the zeta potential of resulting nanoparticles.

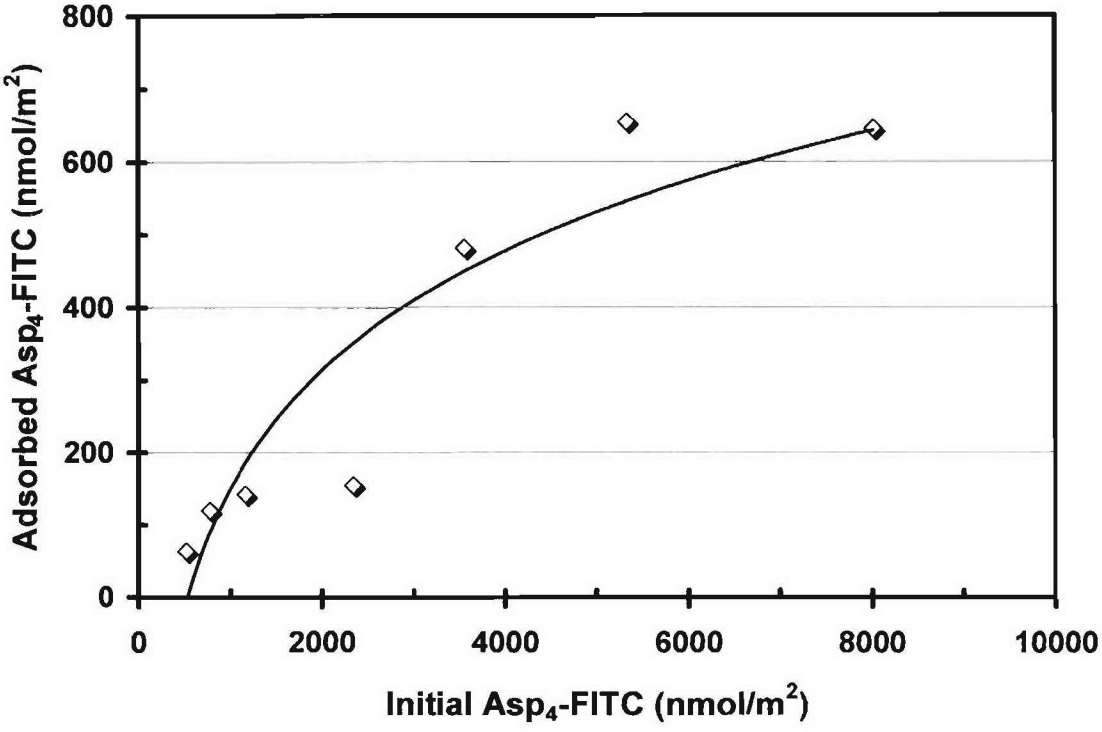


Figure 5. Absolute adsorption of Asp_4-FITC conjugate onto hydroxyapatite substrate in vitro.

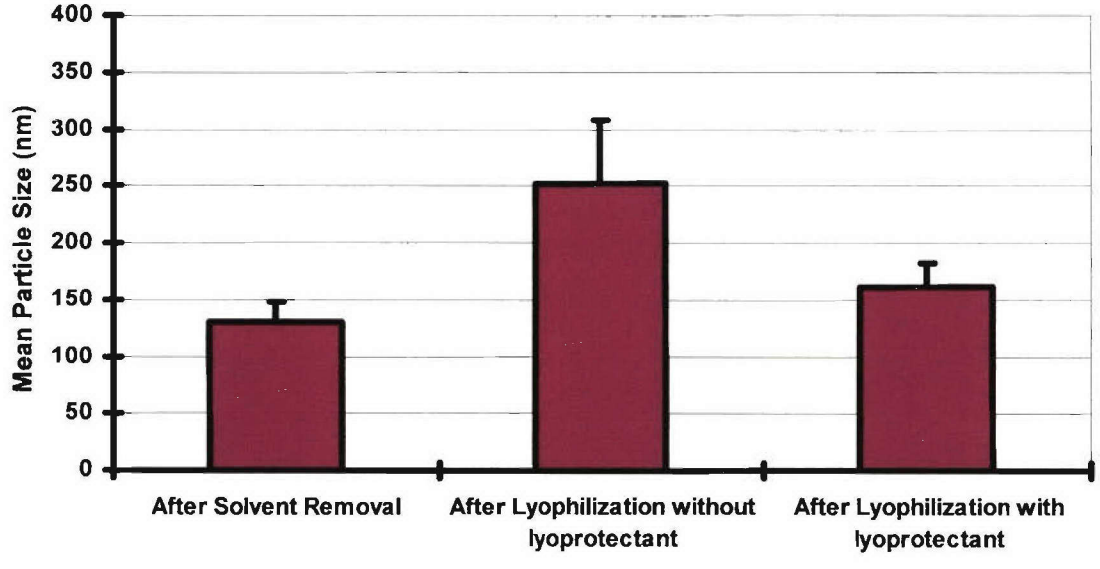


Figure 6. Effect of lyoprotectant on mean particle size of PLGA nanoparticles.

Appendix 2
Quarterly Report 4

Quarterly Report 4

1. Contract No. W81XWH-05-C-0004 2. Report Date 17 February 2006

3. Reporting period from 1 November 2005 to 31 January 2006

4. PI Neal Vail, Ph.D. 5. Telephone No. 210 522 5351

6. Institution Southwest Research Institute

7. Project Title Targeted Bone Cancer Therapy

8. Current staff, with percent effort of each on project.

Neal Vail (PL3)	15%	Senior Tech	1%
PL1	10%	Clerical	2%

9. Contract expenditures to date (as applicable):

	This Qtr / Cumulative		This Qtr / Cumulative
Personnel	$13,039.20 / $57,952.32	Travel	$0 / $0
Fringe Benefits	$6,258.81 / $27,817.11	Equipment	$0 / $0
Supplies	$8,150.51 / $23,995.19	Other	$0 / $0

	This Qtr / Cumulative
Subtotal	$27,448.52/ $109,764.62
Indirect Costs	$24,060.39 / $106,780.84
Fee	$4,017.63 / $16,877.80
Total	$55,526.54 / $133,423.26

10. Comments on administrative and logistical matters.

There have been no administrative or logistical problems encountered as yet.

11. Use additional pages as necessary to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract.

See attached pages.

12. Use additional pages to present a brief statement of plans or milestones for the next quarter. Complete ligand attachment. Develop methods of encapsulating quantum dots for fluorescent imaging of nanoparticles. Start protocol development for encapsulation of proteasome inhibitors. Start ex-vivo perfusion studies to study nanoparticle uptake as a function of ligand content, pending review of protocol by sponsor.

Project Overview

Multiple myeloma is the second most common adult hematologic malignancy. The disease is uniformly fatal with 80% of patients suffering devastating and progressive bone destruction. SwRI has teamed with the University of Texas Health Science Center at San Antonio, OsteoScreen, and Walter Reed Army Medical Center to develop and evaluate bone-targeting nanocarriers that can preferentially accumulate in the skeleton and local release proteasome inhibitors, such as MG-262, to impair the capacity of myeloma cells to survive in vivo, thereby reducing the formation and growth of tumor-induced lytic bone lesions.

The following tasks were investigated during the recent reporting period

- Nanoparticle preparation protocol.
- Alternative nanoparticle preparation protocol.
- Synthesis of PLGA-b-PEG-Ligand.

Each of these items is discussed in more detail in the following sections.

Cryo- and lyo-stability of Nanoparticles

We previously reported the demonstration of two different methods for producing nanoparticles: 1. high-shear emulsification, and, 2. nanoprecipitation. Both of these methods produce narrowly dispersed particles with particle sizes achievable in the range of about 100-200nm, depending on the nanoparticle formulation. We further reported on our efforts to prepare storage-stable versions of these nanoparticles by lyophilization. However, the redispersibility of these lyophilized materials was compromised, resulting in highly aggregated particles. We examined the use of lyoprotectants, typically low-molecular weight sugars, and showed we could maintain the intrinsic particle size of PLGA nanoparticles through the lyophilization process. In this past quarter we have been examining the lyostabilization of PEG-containing nanoparticles.

We selected a polymer composition of 95% wt. PLGA / 5% wt. PLA-PEG as the formulation of our PEGylated nanoparticles. This composition is based on an analysis of the minimum amount of PEG required to achieve full coverage of the nanoparticle surface. Examination of the lyostability of these particles indicated they were somewhat less sensitive to the process yielding dispersions with only small changes in the average particle size, but with noticeably broader distributions (Figure 1). Increasing amounts of sucrose in the final nanoparticle preparation re-established the narrow the particle size distribution (Figure 2).

Alternative Nanoparticle Preparation

To date we have been removing polymer solvent used in the nanoparticle preparation by rotory evaporation. This method is suitable for small volumes, although we have seen some deleterious effects on the particle size distribution, such as broadening of the

distribution. Also, for more rigorous solvents, such as dimethyl acetimide (DMA), DMSO, or dimethyl formamide (DMF), solvent removal by rotory evaporation can be problematic, if not impossible, due to the low glass transition temperature of the polymer nanoparticles. Once solvent is removed we then separate the particles from their natant fluid by ultracentrifugation and then resuspend the particles in clean fluid. This cleaning process is very sensitive to the centrifuge conditions and can also have very dramatic effects on the particles, from complete solidification to broadening of the distribution.

To overcome some of these problems we have experimenting with cross-flow filtration equipment as a means to do both solvent removal and concentrate the nanoparticles for resuspension in clean fluids. We examined its use with particles prepared by nanoprecipitation and found it to be very effective both in removing solvent in a timely fashion and providing an easily redispersible nanoparticle cake. We are presently conducting further experiments to incorporate cross-flow filtration into our nanoparticle formulation protocol.

Synthesis of PLA-b-PEG-Ligand

The bone-targeting capability of our nanoparticles will be provided by covalently attaching previously identified ligands to the nanoparticles surfaces via an activated polyethylene glycol (PEG) linker. Our approach to preparing this linker is to start with a bifunctional PEG and selectively attach to the respective ends either the bone-targeting ligand or a polylactide polymer.

As reported previously, we were able to procure the required bifunction PEG from a commercial source. This material has the form HO-PEG-NH_2 with the PEG segment being approximately 3400 molecular weight. The synthetic approach is to couple the PLA segment to the PEG block via the HO- moiety, leaving the $-NH_2$ moiety for attaching the ligand.

The first step is to attach PLA to the bifunctional PEG using ring-opening polymerization in the presence of a coordination catalyst as illustrated in Figure 3 (see Scheme 1). The amine group (-NH_2) is protected during the polymerization. Using tin 2-ethylhexanoate as a catalyst and lactide monomer, we prepared PLA-b-PEG-NH2 block copolymer and confirmed the structure and composition of the recovered product by ^{1}H-NMR (data not shown). In one example, the resulting molecular weight of the PLA block was estimated to be about 25,500. The PLA block length can be altered by adjusting the catalyst content using an inverse relationship based on the polymerization kinetics. We obtained PLA blocks of approximately 44,000 molecular weight by simply reducing the amount of catalyst. This particular block length is essentially equivalent to the molecular weight of the PLGA polymers used in the base nanoparticle formulation. We have since prepared approximately 1g of this PLA-b-PEG-NH_2 material.

Bone-targeting ligands are covalently attached to the PEG linker via a thiol coupling to a maleimide group (see Scheme 3, Figure 3). The maleimide group is introduced to the –NH2 terminus of the previously prepared PLA-b-PEG material by reaction with 3-

maleimidopropionic acid N-hydroxysuccinimide ester (MPS). We found that the maleimide functionalization could not be achieved using the PLA-b-PEG-NH_2 starting material. Alternatively, we found it necessary to first attach the maleimide group to the HO-PEG-NH_2, followed by PLA attachment to the HO-PEG-Maleimide by the previously described ring-opening polymerization. Structures were confirmed at each of the key synthesis points by ^{1}H-NMR. The typical product had a PLA block size of about 52,000 molecular weight.

Our next step in this development is to attach the selected bone-targeting ligands to these maleimide-functionalized block copolymers. Both of the selected ligands, amino methylene bisphosphonate (aMBP) and an aspartic acid oligomer (Asp_n), have an amino terminus. This is converted to thiol by reaction with Traut's Regeant (2-iminothiolane, see Scheme 2, Figure 3). This thiol analogue then reacts with the maleimide moiety of the block copolymer to yield a covalently attached ligand. This work is in progress.

Other Matters

The principal investigator has submitted an abstract to and will be attending the DoD-USAMRMC/PRMRP Military Health Research Forum scheduled for May 1 – 5, in San Juan, Puerto Rico.

The principal investigator was invited to give a talk at the Particles 2006 – Medical/Biochemical Diagnostic, Pharmaceutical, and Drug Delivery Applications of Particle Technology Forum scheduled for May 13 –16, in Orlando, FL and will presenting work from this program.

Next Steps

Complete ligand attachment. Develop methods of encapsulating quantum dots for fluorescent imaging of nanoparticles. Start protocol development for encapsulation of proteasome inhibitors. Start ex-vivo perfusion studies to study nanoparticle uptake as a function of ligand content, pending review of protocol by sponsor.

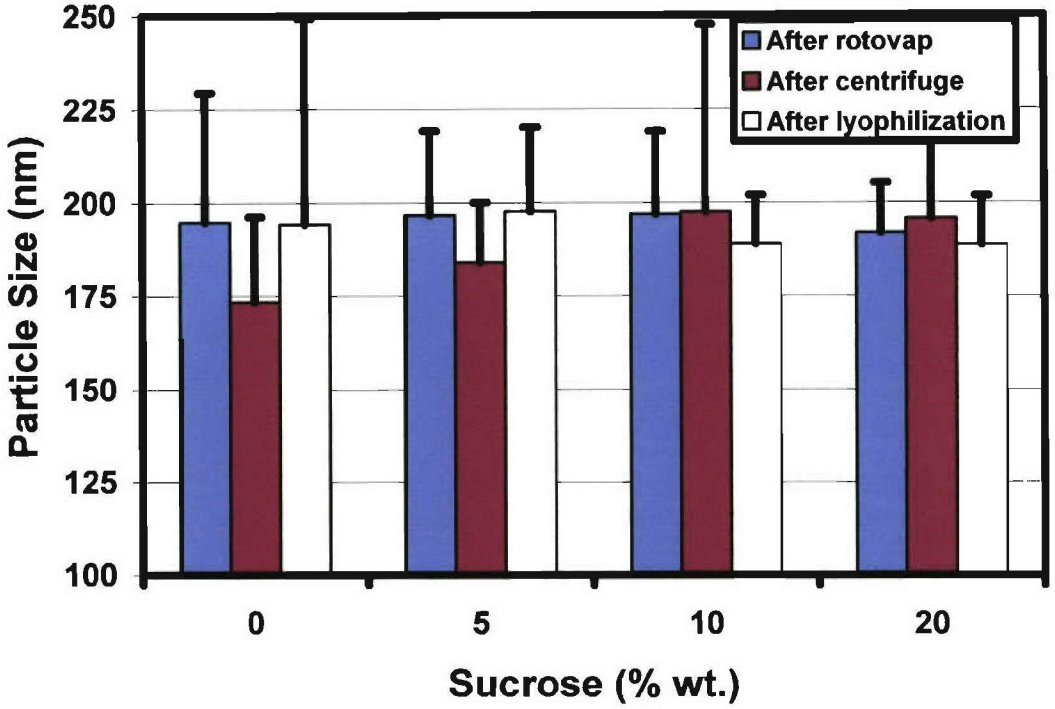


Figure 1. Effect of processing steps and the addition of sucrose as a lyoprotectant on particle size distribution of 95/5 w/w PLGA/PLA-PEG nanoparticles.

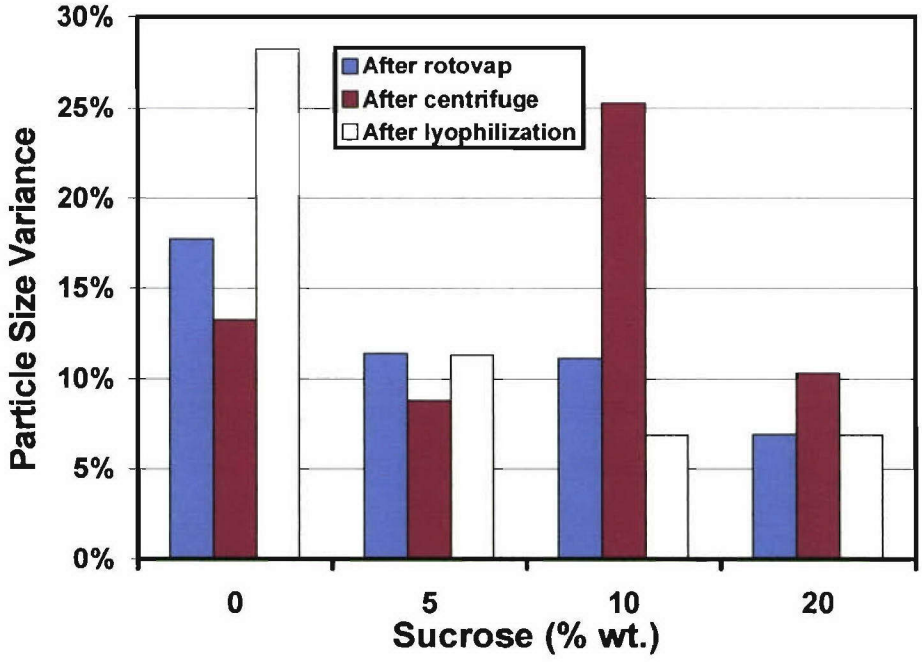
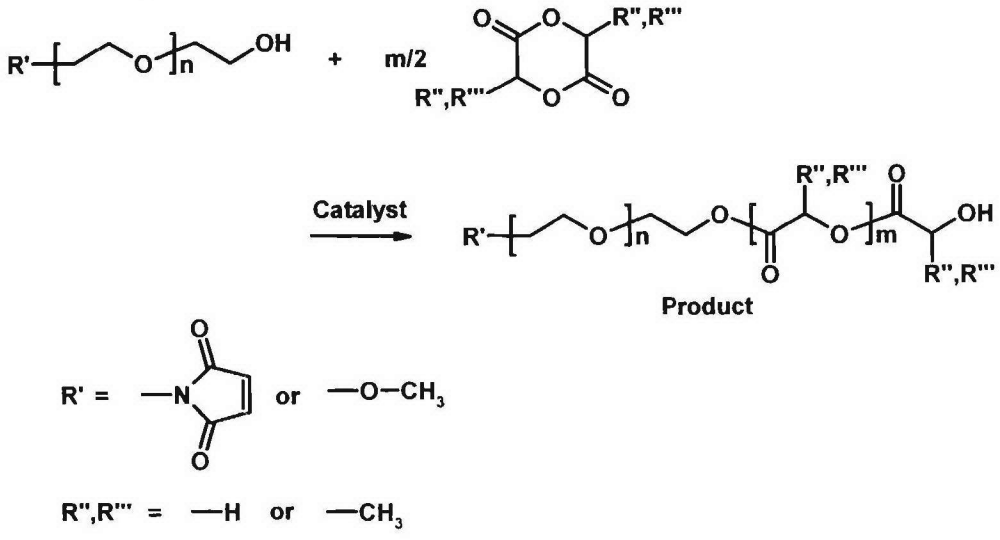
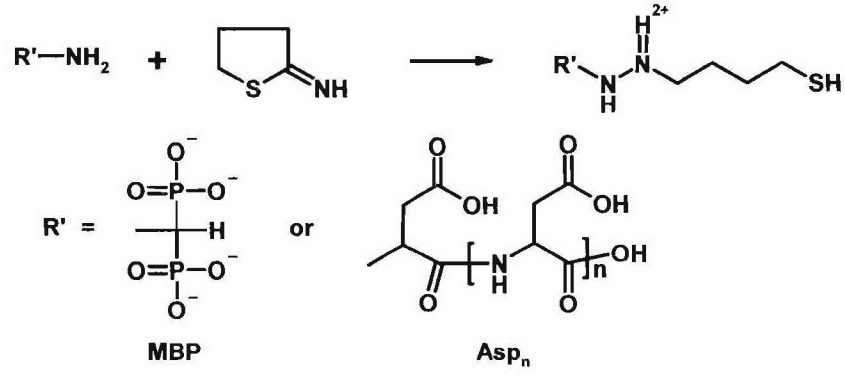


Figure 2. Effect of lyoprotectants on the particle size distribution of 95/5 w/w PLGA/PLA-PEG nanoparticles. The particle size distribution is narrowed with increasing sucrose content. A minimum of about 5% wt. sucrose is needed to maintain narrow particle size distribution during lyophilization.

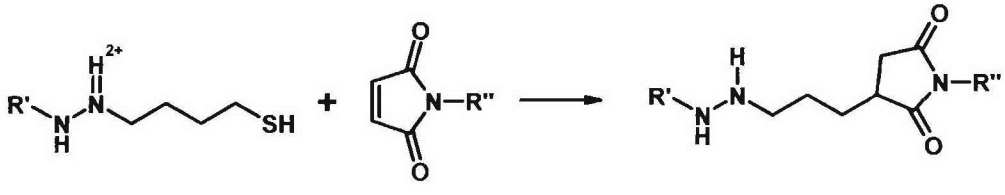
Scheme 1 - Synthesis of Polyethylene glycol - polylactide-co-galactide Block Copolymers



Scheme 2 - Thiolation of Amino-terminated Bone-targeting Ligands



Scheme 3 - Conjugation of Thiolated Bone-targeting Ligands to PEG-Lactide Block Copolymers



R' = See Scheme 2 above
R" = Product from Scheme 1 above

Figure 3. Synethic schemes for the preparation of PEGylated polylactide block copolymers (Scheme 1), thiolation of amino-terminated bone-targeting ligands (Scheme 2), and, conjugation of thiolated bone-targeting ligands to maleimide-functionalized PEGylated polylactide block copolymers (Scheme 3).